Supplemental Information

An Interleukin-33-mast cell-interleukin-2 axis suppresses papain-induced allergic inflammation by promoting regulatory T cell numbers

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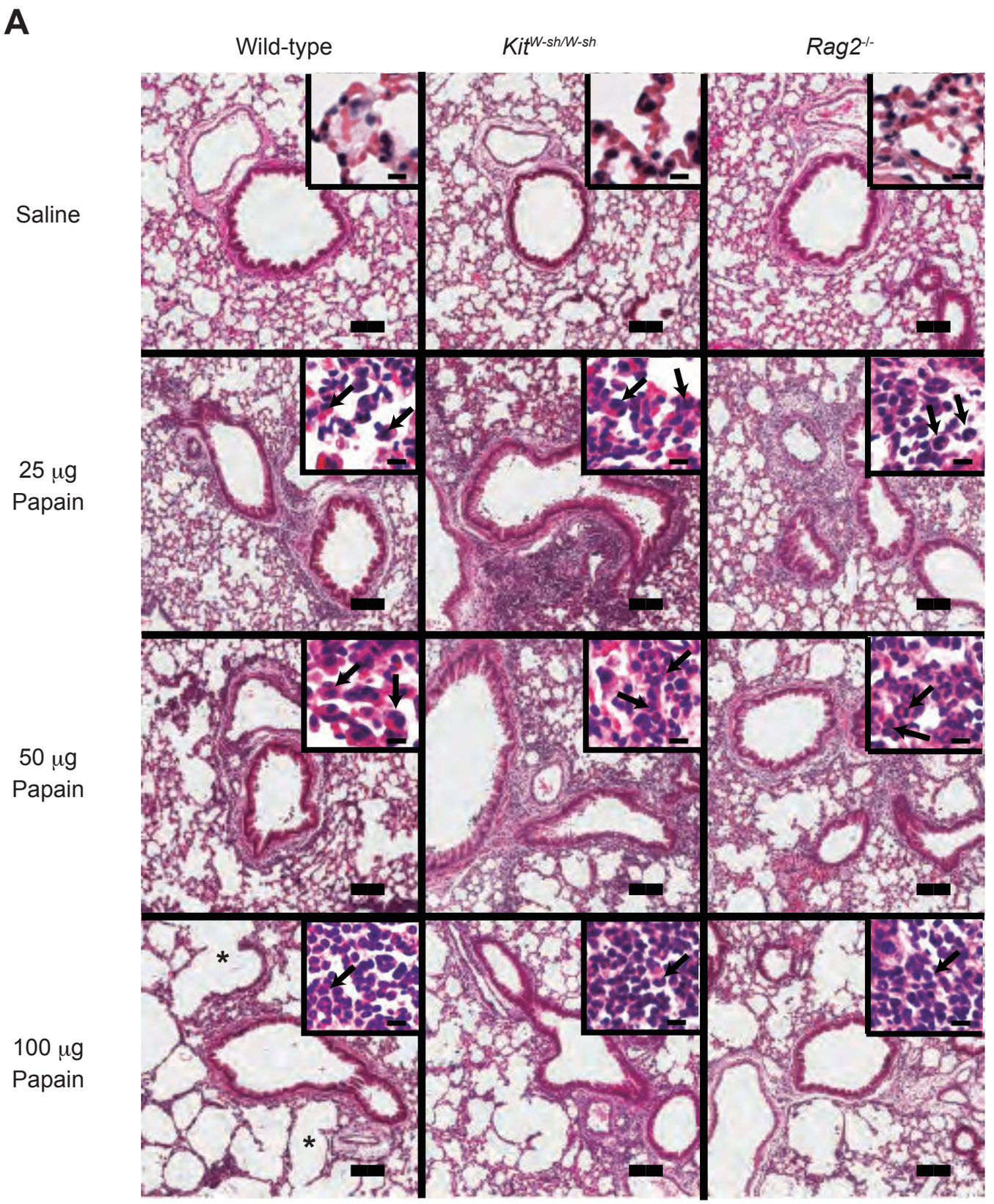

A
Wild-type
Kit W-sh/W-sh
Rag2 -/-
Saline
25 μg Papain
50 μg Papain
100 μg Papain
*
*

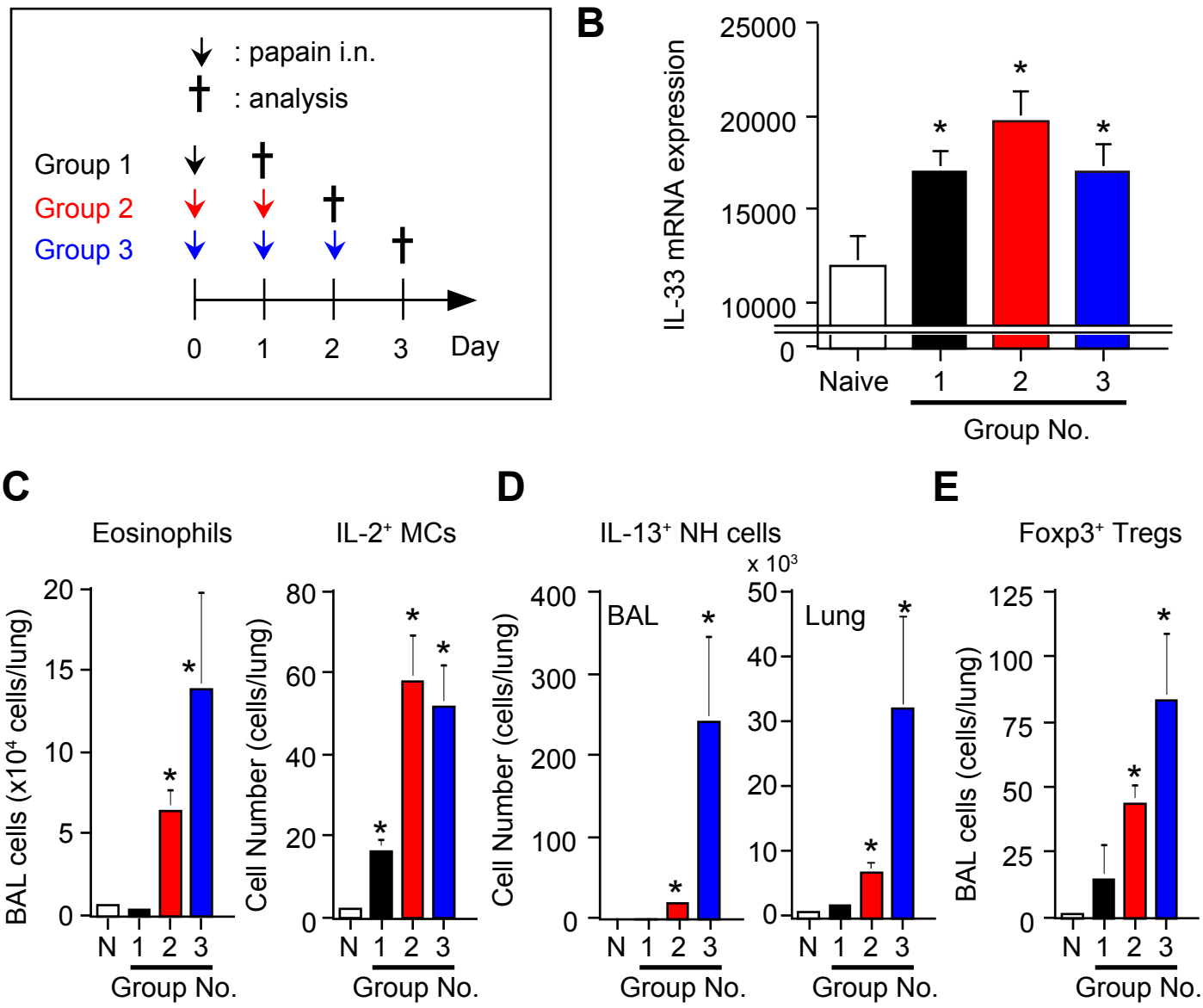

: papain i.n.
: analysis
Group 1
Group 2
Group 3
0
1
2
3
Day
B
IL-33 mRNA expression
25000
20000
15000
10000
0
Naive
1
2
3
Group No.
C
Eosinophils
BAL cells (x10⁴ cells/lung)
20
15
10
5
0
N 1 2 3
Group No.
IL-2⁺ MCs
Cell Number (cells/lung)
80
60
40
20
0
N 1 2 3
Group No.
D
IL-13⁺ NH cells
Cell Number (cells/lung)
BAL
400
300
200
100
0
N 1 2 3
Group No.
Lung
x 10³
50
40
30
20
10
0
N 1 2 3
Group No.
E
Foxp3⁺ Tregs
BAL cells (cells/lung)
125
100
75
50
25
0
N 1 2 3
Group No.

Figure S1 (related to Figure 1). IL-33 mRNA expression and the number of eoshinophils, Tregs, MCs and NH cells in the lungs or BALFs after papain inhalation.

(**A**) C57BL/6J-wild-type, -*Kit*$^{W\text{-}sh/W\text{-}sh}$ and -*Rag2*$^{-/-}$ mice were treated intranasally with low-dose (25 μg) or high-dose (50-100 μg) papain or saline. Lungs were harvested 24 h after the last inhalation of papain or saline. Note the enlarged air spaces (some marked: *) in the lungs of wild-type mice treated with 100 μg papain. Sections of the lungs were stained with H & E. Representative results of the lung histology in each group are shown. Scale bars = 200 μm and 10 μm (in magnified pictures).

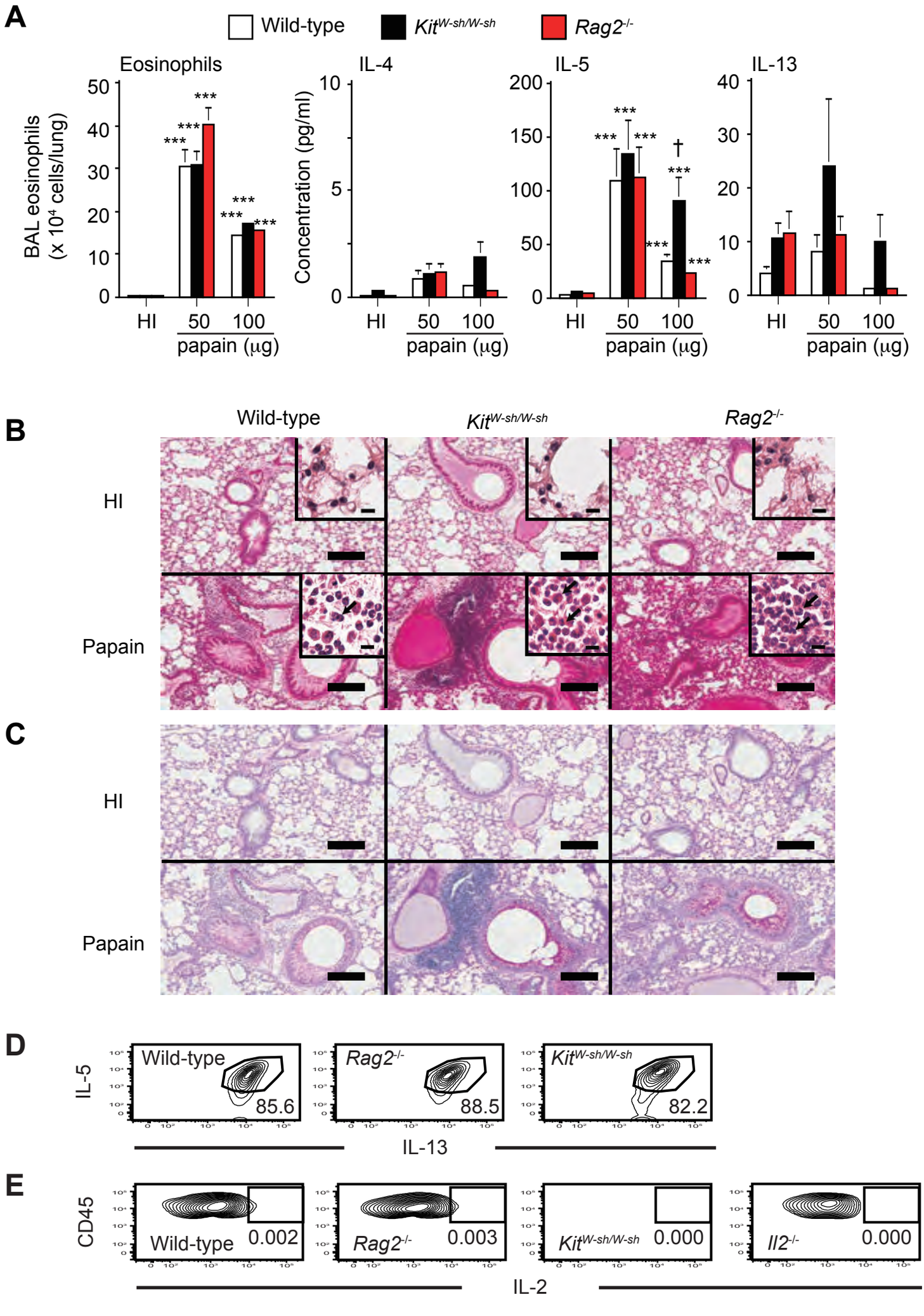
(**B**) C57BL/6N-wild-type mice were treated with 25 μg papain intranasally as a scheme in a right box. The lungs were harvested 24 h after the 1st (Group 1), 2nd (Group 2) and 3rd (Group 3) papain inhalation. The expression levels of IL-33 mRNA were determined by quantitative PCR. Data show the mean + SEM (n=5). * $p<0.05$ vs. naïve mice (N).

(**C**) The numbers of eosinophils and CD45$^+$c-Kit$^+$ FcεRIα$^+$ IL-2$^+$ MCs in the lungs from C57BL/6-wild-type mice.

(**D**) The numbers of lin$^-$ Sca1$^+$ c-Kit$^+$ tdTomato$^+$ NH cells in the BALFs (D) and lungs (E) from C57BL/6-IL-13-tdTomato reporter mice. IL-13$^+$ ILC2 also expressed IL-5 (please see Fig. S2D).

(**E**) The numbers of CD45$^+$ CD3$^+$ CD4$^+$ CD25$^+$ GFP$^+$ Tregs in the BALFs from C57BL/6-Foxp3-GFP reporter mice.

The percentage of CD3$^+$ CD4$^+$ CD25$^+$ GFP$^+$ Tregs or lin$^-$ Sca1$^+$ c-Kit$^+$ tdTomato$^+$ NH cells in CD45$^+$ cells of the BALFs and CD45$^+$c-Kit$^+$ FcεRIα$^+$ IL-2$^+$ MCs of the lungs were determined by flow cytometry, then, the number of GFP$^+$ Tregs, tdTomato$^+$ NH cells or IL-2$^+$ MCs was calculated as below: Total number of BALFs or lungs x % CD45$^+$ CD3$^+$ CD4$^+$ CD25$^+$ GFP$^+$ Tregs, % lin$^-$ Sca1$^+$ c-Kit$^+$ tdTomato$^+$ NH cells or % CD45$^+$ c-Kit$^+$ FcεRIα$^+$ IL-2$^+$ MCs. Data show the mean + SEM (n=5). * $p<0.05$ vs. naïve mice.


A
Wild-type
Kit W-sh/W-sh
Rag2 -/-
Eosinophils
IL-4
IL-5
IL-13
BAL eosinophils (x 10⁴ cells/lung)
Concentration (pg/ml)
HI
50
100
papain (μg)
***
†
B
Wild-type
Kit W-sh/W-sh
Rag2 -/-
HI
Papain
C
HI
Papain
D
Wild-type
Rag2 -/-
Kit W-sh/W-sh
85.6
88.5
82.2
IL-5
IL-13
E
CD45
Wild-type
0.002
Rag2 -/-
0.003
Kit W-sh/W-sh
0.000
Il2 -/-
0.000
IL-2

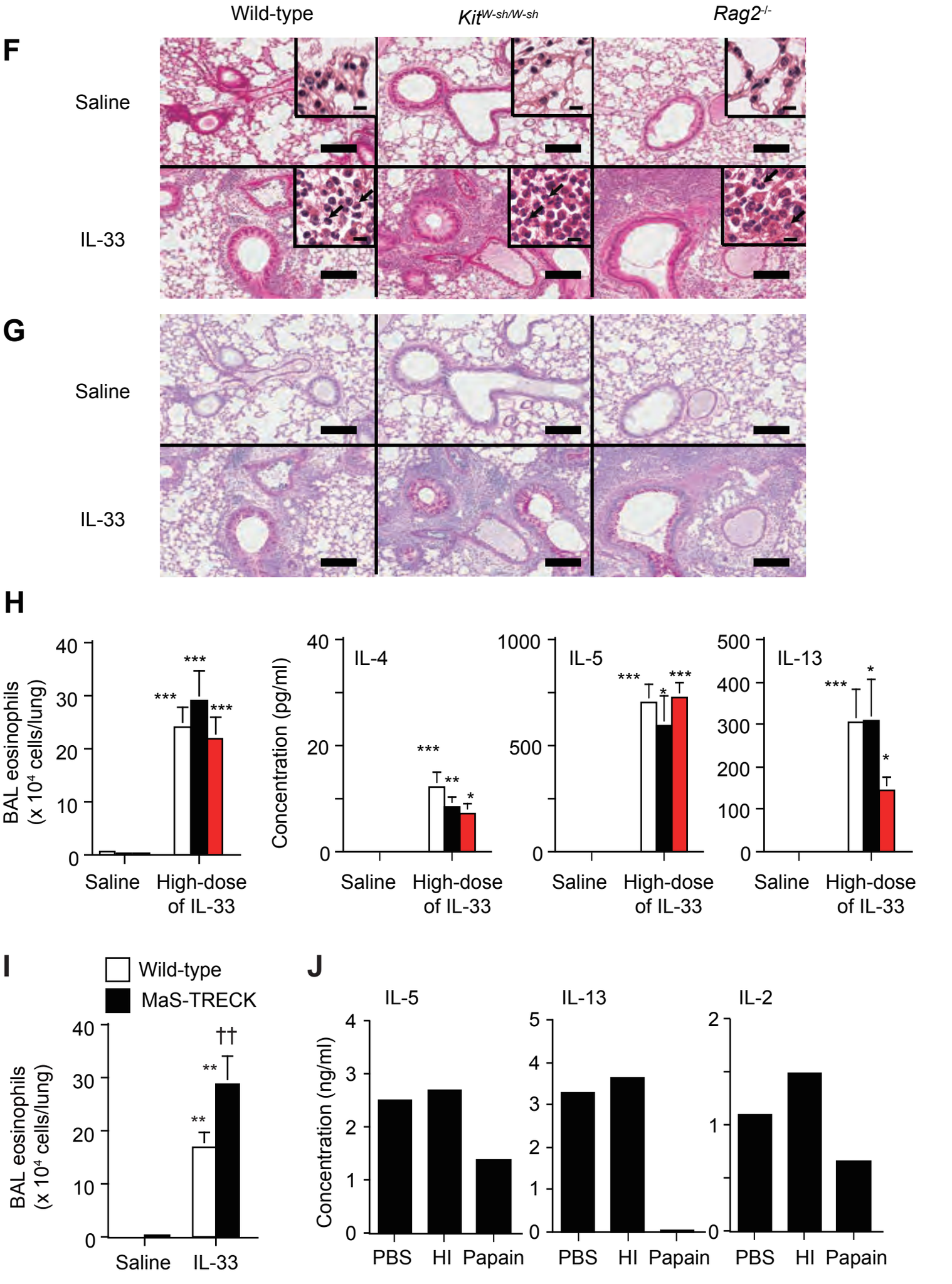

F
Wild-type
Kit W-sh/W-sh
Rag2 -/-
Saline
IL-33
G
Saline
IL-33
H
BAL eosinophils (x 10⁴ cells/lung)
Saline
High-dose of IL-33
Concentration (pg/ml)
IL-4
IL-5
IL-13
I
Wild-type
MaS-TRECK
BAL eosinophils (x 10⁴ cells/lung)
Saline
IL-33
J
IL-5
IL-13
IL-2
Concentration (ng/ml)
PBS
HI
Papain

Figure S2 (related to Figure 2). Papain- and IL-33-induced airway inflammation in MC- and Rag2-deficient mice.

(**A**) Numbers of eosinophils and amounts of IL-4, IL-5 and IL-13 in BALF from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Rag2*$^{-/-}$ mice 24 h after the final inhalation of high-dose papain (50 or 100 μg; n=10-21) or heat-inactivated papain (HI; 100 μg, n=5-8).

(**B, C**) Twenty-four h after the last inhalation of 25 μg of papain or heat-inactivated papain (HI) or saline, lungs were harvested from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Rag2*$^{-/-}$ mice.

(**B**) H&E staining. (**C**) PAS staining. Representative results of the lung histology in each group are shown.Scale bars = 200 μm and 10 μm (in magnified pictures). Arrow = eosinphil.

(**D**) C57BL/6J-wild-type mice, *Rag2*$^{-/-}$ mice and mast cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were treated with 25 μg papain intranasally. The BALFs were collected from these mice 24 hours after the last papain inhalation. Then, cells were stimulated with PMA + ionomycin overnight. After the cultivation, intracellular IL-5 and IL-13 expression in CD45$^+$ lin$^-$ Sca1$^+$ CD25$^+$ KLRGR1$^+$ NH cells was determined by flow cytometry. The data show a representative results from one of the 4-5 mice analyzed, each of which gave similar results. NH cells were hardly detectable in the BALF from saline-treated mice (data not shown).

(**E**) C57BL/6J-wild-type mice, *Rag2*$^{-/-}$ mice, mast cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Il2*$^{-/-}$ mice were treated with papain intranasally. The lungs were collected from these mice 24 hours after the last papain inhalation. Then, cells were stimulated with PMA + ionomycin in the presence of monensin for 3 hours. After the cultivation, intracellular IL-2 expression in CD45$^+$ c-Kit$^+$ FcεRIα$^+$ MCs was determined by flow cytometry. The data show a representative results from one of the 4-5 mice analyzed, each of which gave similar results. IL-2$^+$ MCs were hardly detectable in the lungs and BALFs from saline-treated mice (data not shown).

(**F, G**)Twenty-four h after the last inhalation of 0.1 μg of IL-33 or saline, lungs were harvested from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Rag2*$^{-/-}$ mice. (**F**) H&E staining. (**G**) PAS staining. Representative results of the lung histology in each group are shown.Scale bars = 200 μm and 10 μm (in magnified pictures). Arrow = eosinphil.

(**H**) Numbers of eosinophils and levels of IL-4, IL-5 and IL-13 in BALF from wild-type mice, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Rag2*$^{-/-}$ mice 24 h after the final inhalation of high-dose IL-33 (5 μg) (saline, n=4-6; and IL-33, n=11-15). The data show the mean + SEM. *P<0.05, ** P<0.01 and ***P<0.005 vs. HI or saline, and †P<0.05 vs. papain-treated wild-type mice.

(**I**) The numbers of eosinophils in BALF, the proportion of CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells in thoracic LNs of DT-injected wild-type or MaS-TRECK mice after inhalation of 0.1 μg of IL-33 (saline: n = 5-6; IL-33: n = 8-10). The data are the mean + SEM. **P<0.01 vs. saline, and ††P<0.01 vs. wild-type mice.

(**J**) Recombinant mouse IL-5, IL-13 and IL-2 were incubated with or without papain at 37°C for 30 min. After stopping the reaction by addition of protease inhibitor solution, the concentrations of IL-5, IL-13 and IL-2 were measured by ELISA. Data show representative results from three-independent experiments. HI = heat-inactivated papain.

Figure S3

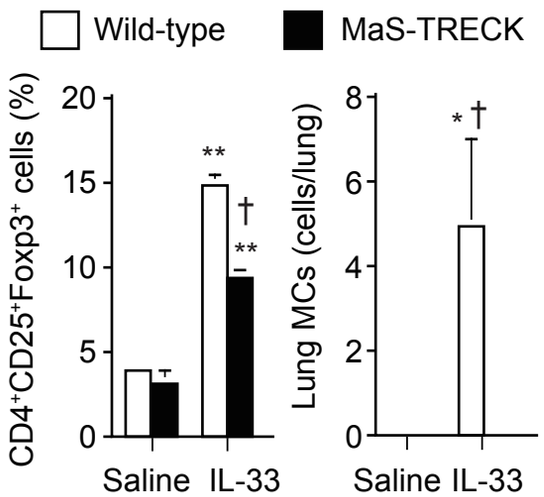
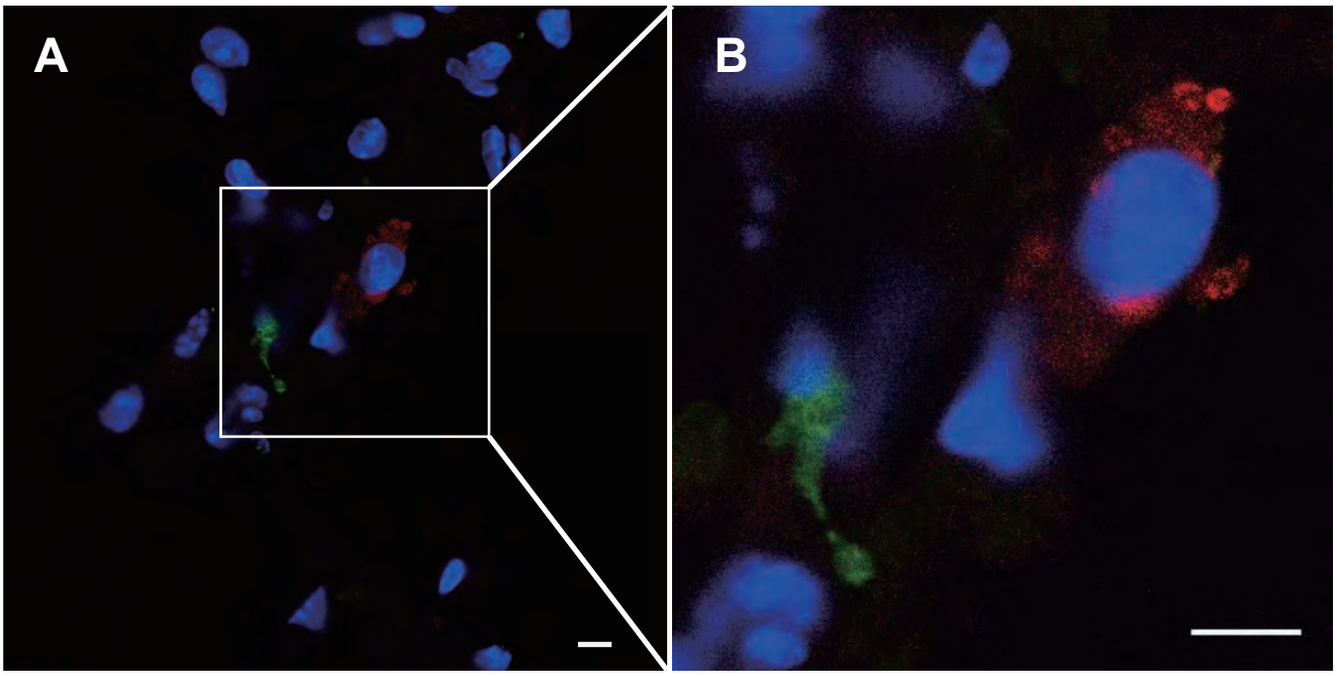


Figure S3 (related to Figure 3). Exacerbated IL-33-mediated airway eosinophilia in MC-depleted MaS-TRECK mice.

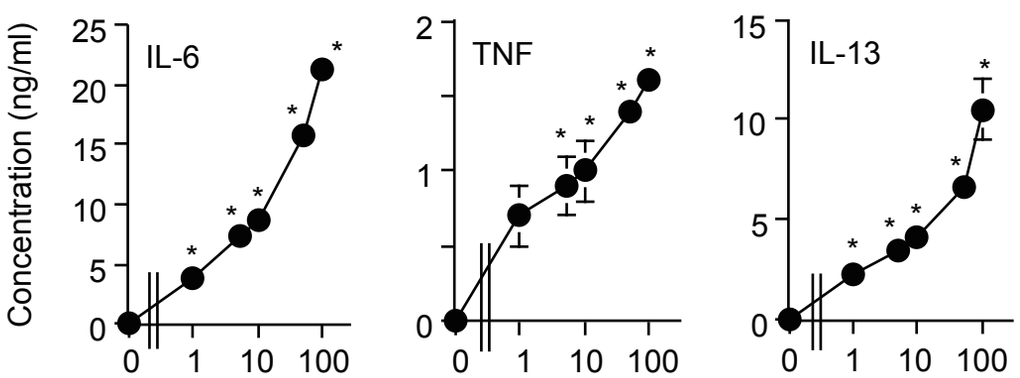
The numbers of eosinophils in BALF, the proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in thoracic LNs,and the numbers of MCs in the lungs of DT-injected wild-type or MaS-TRECK mice after inhalation of 0.1 μg of IL-33 (saline: n = 5-6; IL-33: n = 8-10) as shown in Figure S2I. The data are the mean + SEM.

*P<0.05 and **P<0.01 vs. saline, and †P<0.05 vs. wild-type mice.

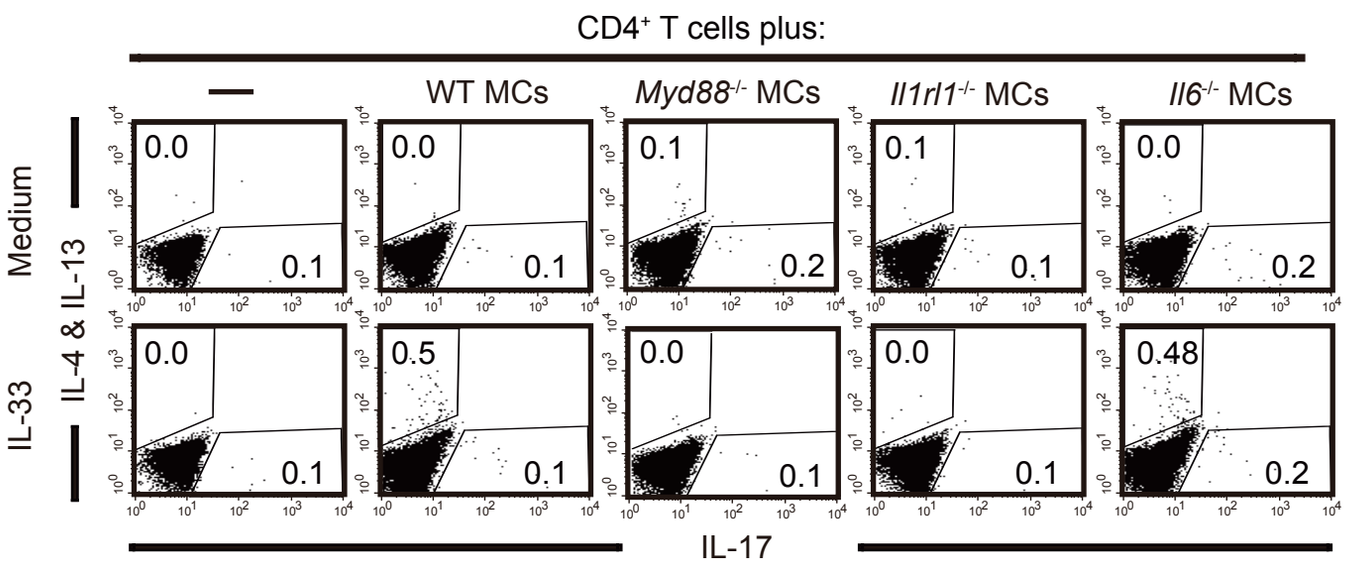


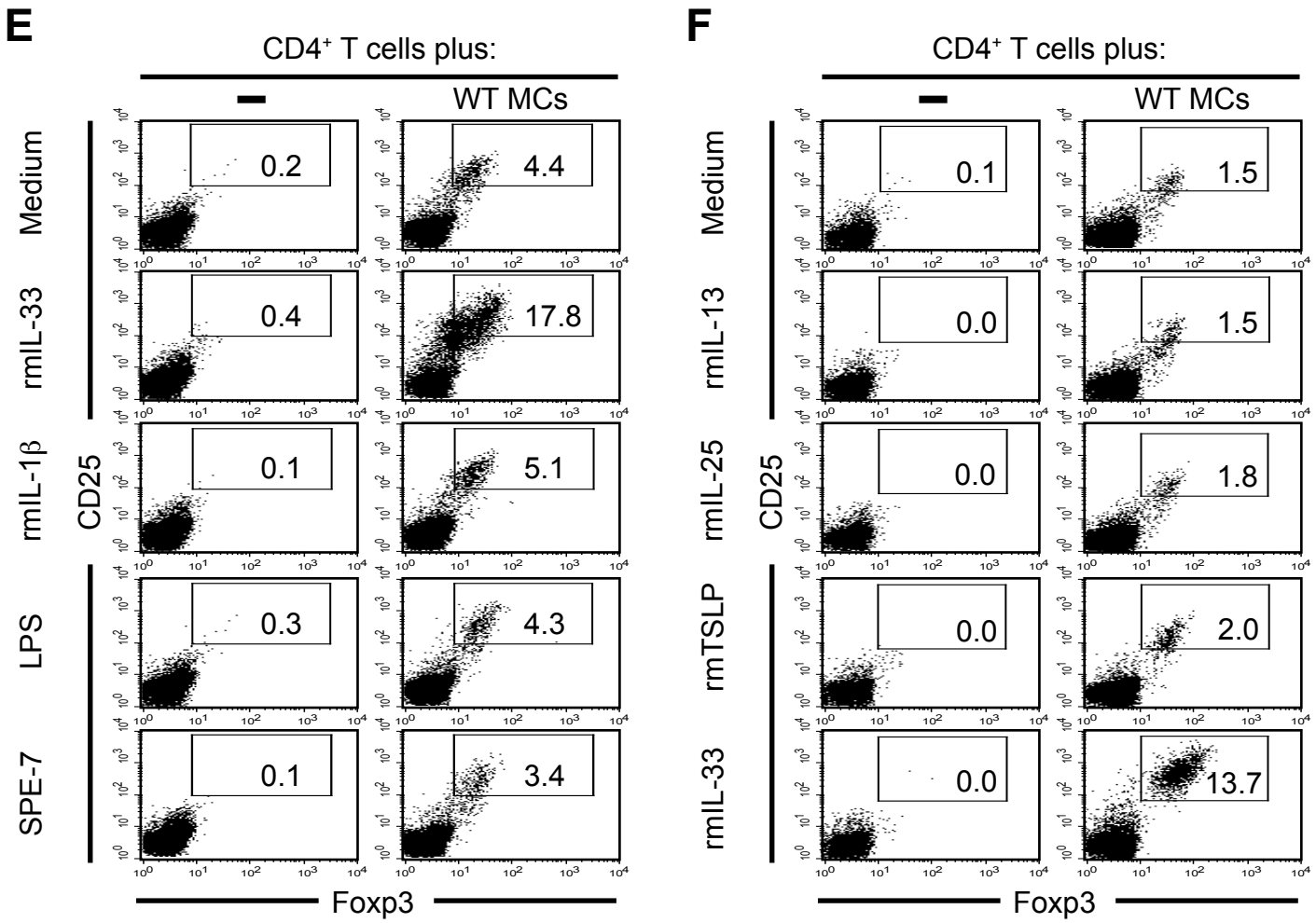
Green = Foxp3⁺ Treg, Red = MC, and Blue = Hoechst

C



D




E
CD4+ T cells plus:
–
WT MCs
Medium
rmIL-33
rmIL-1β
LPS
SPE-7
CD25
Foxp3
0.2
4.4
0.4
17.8
0.1
5.1
0.3
4.3
0.1
3.4
F
CD4+ T cells plus:
–
WT MCs
Medium
rmIL-13
rmIL-25
rmTSLP
rmIL-33
CD25
Foxp3
0.1
1.5
0.0
1.5
0.0
1.8
0.0
2.0
0.0
13.7

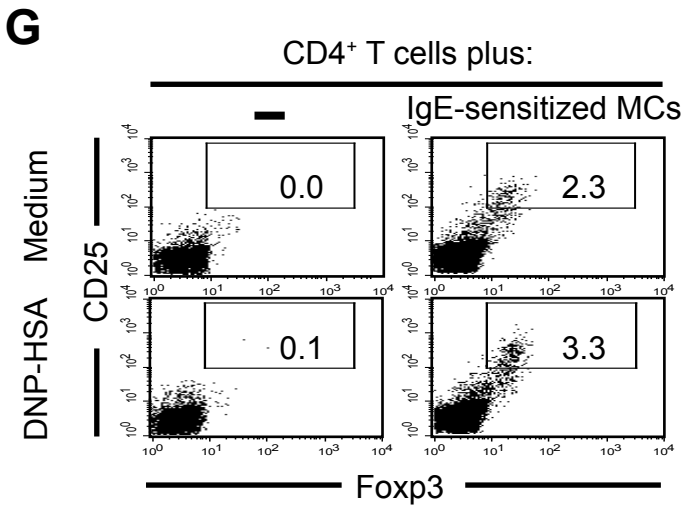

G
CD4+ T cells plus:
–
IgE-sensitized MCs
Medium
DNP-HSA
CD25
Foxp3
0.0
2.3
0.1
3.3

Figure S4 (related to Figure 4). IL-33-stimulated mast cells promote expansion of Treg cells.

(**A, B**) Lungs were harvested from Foxp3-EGFP reporter mice 24 h after the final inhalation of papain (25 μg). Frozen lung sections were stained with Hoechst, Avidin-Texas Red and Alexa488 anti-GFP Ab. Scale bars = 5 μm. Representative results of the lung histology in each group are shown.

(**C**) Naïve mast cells (MCs: bone-marrow-derived cultured mast cells, 1 x 10^6 cells/well in 24-well plates) were stimulated with various concentrations of IL-33 for 24 h. Concentrations of IL-6, TNF, and IL-13 in the culture supernatants were measured by ELISA. Data show the mean ± SEM from 3-5 batches of MCs per experiment and are representative results from one of the two independent experiments performed, each of which gave similar results.

*P<0.05 vs. no cytokine (medium only).

(**D**) Mouse whole $CD4^+$ T cells (2 x 10^6 cells/well) were cultured with or without wild-type (WT) bone-marrow-derived cultured mast cells (MCs), *Myd88*$^{-/-}$ MCs, *Il1rl1*$^{-/-}$ MCs or *Il6*$^{-/-}$ MCs (5 x 105 cells/well) in the presence or absence of 100 ng/ml rhIL-33 for 3 days.

The proportions of IL-4^+ IL-13^+ Th2 cells and IL-17^+ Th17 cells among c-kit-negative $CD4^+$ T cells were assessed by flow cytometry. Data show representative results from 3-5 batches of MCs per experiment from the 2-3 independent experiments performed, each of which gave similar results..

(**E, F**) $CD4^+$ T cells (2 x 10^6 cells/well) were co-cultured with or without wild-type (WT) bone-marrow-derived culturedmast cells (MCs : 5 x 10^5 cells/well) in the presence or absence of 100 ng/ml rmIL-33, rmIL-1β, rmIL-13, rmIL-25 or rmTSLP, 1 μg/ml LPS or 1 μg/ml monomeric IgE (SPE-7) for 3 days in 24-well plates.

(**G**) $CD4^+$ T cells (2 x 10^6 cells/well) were co-cultured with and without IgE-sensitized WT MCs (5 x 10^5 cells/well) in the presence and absence of 100 ng/ml DNP-HSA for 3 days in 24-well plates. (**E-G**) The proportion of $CD25^+Foxp3^+$ Treg cells among c-kit-negative $CD4^+$ T cells was assessed by flow cytometry. Data show representative results from 3-5 batches of MCs per experiment and from at least two independent experiments, each of which gave similar results.

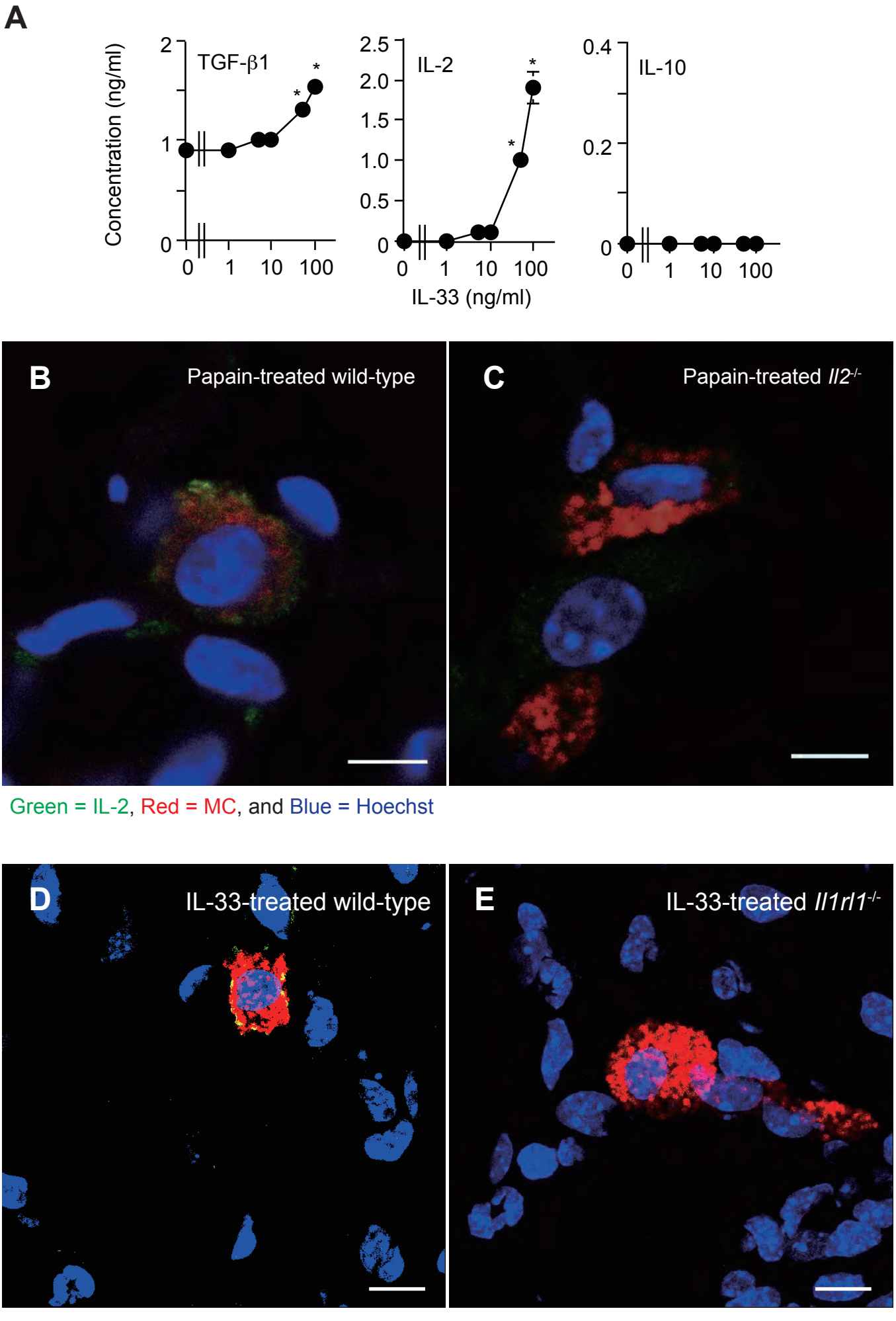

A
Concentration (ng/ml)
TGF-β1
IL-2
IL-10
IL-33 (ng/ml)
B
Papain-treated wild-type
C
Papain-treated Il2-/-
Green = IL-2, Red = MC, and Blue = Hoechst
D
IL-33-treated wild-type
E
IL-33-treated Il1rl1-/-
Green = IL-2, Red = MC, and Blue = Hoechst

Figure S5 (related to Figure 6). IL-33 induces IL-2 production by mast cells.

(**A**) Naïve mast cells (MCs: bone-marrow-derived cultured mast cells, 1 x 10^6 cells/well in 24-well plates) were stimulated with various concentrations of IL-33 for 24 h. Concentrations of TGF-β1, IL-2 and IL-10 in the culture supernatants were measured by ELISA. Data show the mean ± SEM from 3-5 batches of MCs per experiment and are representative results from one of the two independent experiments performed, each of which gave similar results.

*P<0.05 vs. no cytokine (medium only)

(B, C) Lungs were harvested from C57BL/6J-wild-type mice and -*Il2*$^{-/-}$ mice 24 h after the final inhalation of papain (25 μg). Frozen lung sections were stained with Hoechst, Avidin-Texas Red and anti-mouse IL-2 Ab. Scale bars = 5 μm.

(**D, E**) Lungs were harvested from C57BL/6J-wild-type mice and -*Il1rl1*$^{-/-}$ mice 24 h after the final inhalation of IL-33 (0.5 μg). Frozen lung sections were stained with Hoechst, Avidin-Texas Red and anti-mouse IL-2 Ab. Scale bars = 10 μm.

Representative results of the lung histology in each group are shown..

Supplemental Experimental Procedures

Mice

C57BL/6J and C57BL/6N wild-type mice were purchased from Japan SLC, Inc. C57BL/6N-*Il33*$^{-/-}$ and C57BL/6J MaS-TRECK mice were generated as described previously (Oboki et al., 2010; Otsuka et al., 2011). C57BL/6-*Rag2*$^{-/-}$ mice and C57BL/6-*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice were obtained from Taconic Farm (Hudson, NY). C57BL/6-*Il6*$^{-/-}$, C57BL/6-*Il10*$^{-/-}$, C57BL/6-*Par2*$^{-/-}$ and 129×B6-*Tgfb1*$^{+/-}$ mice were obtained from The Jackson Laboratory. C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, which were originally provided by Dr. Peter Besmer (Cornell University Graduate School of Medical Sciences, NY), were backcrossed onto the C57BL/6J background (N10). C57BL/6-*Myd88*$^{-/-}$, C57BL/6-MHC class II (*H2-Aβ1*)$^{-/-}$, C57BL/6-*Il2*$^{-/-}$, C57BL/6-IL-13-tomato reporter, C57BL/6-Foxp3-GFP and C57BL/6-*Il1rl1*$^{-/-}$ mice were kindly provided by Drs. Shizuo Akira (Osaka University, Japan), Satoshi Ishido (RIKEN, Japan), Shigeo Koyasu (Keio University, Japan), Andrew McKenzie (MRC, UK), Xiao-Kang Li (National Research Institute for Child Health

and Development) and Kenji Nakanishi (Hyogo College of Medicine, Japan), respectively. All mice were housed under specific-pathogen-free conditions in an environmentally-controlled clean room at the National Research Institute for Child Health and Development, and The Institute of Medical Science, The University of Tokyo. All experiments were conducted in accordance with the institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation. Age-matched female adult mice (6 to 8 weeks old, except for *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with BMCMCs [~12 weeks old]) were used for experiments.

Bronchoalveolar lavage fluid (BALF)

Briefly, mice were intubated with a 22-G blunt needle (NIPRO, Osaka, Japan), and 1 ml of HBSS with 2% FCS was injected through the needle into the lungs. BALF was collected and centrifuged. The BALF cells were re-suspended in 200 μl of HBSS with 2% FCS, and each cell type was counted with an automated

hematology analyzer, Sysmex XT-1800i (Sysmex Corporation, Hyogo, Japan), according to the manufacturer's instructions.

Histology

Lungs were collected 24 h after the last i.n. administration of papain, heat-inactivated papain, IL-33 or saline, fixed in Carnoy's solution and embedded in paraffin. Three-μm-thick lung sections were prepared and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) or toluidine blue (for detection of MCs).

Depletion of natural helper cells

Briefly, *Rag2*$^{-/-}$ mice were treated with anti-CD25 mAb (PC61) intraperitoneally (250 μg) and i.n. (20 μg) on days -1, 0, 1 and 2 of papain inhalation. These mice were treated with 25 μg papain in 20 μl saline i.n. on days 0, 1 and 2. BALF was collected 24 h after the last inhalation.

Depletion of mast cells

Briefly, C57BL/6J wild-type and C57BL/6J-Mas-TRECK mice were injected with 250 μl of 1 μg/ml diphtheria toxin (DT) (SIGMA-Aldrich) intraperitoneally for five consecutive days. Ten days after the last DT injection, the mice were administered 0.1 μg of IL-33 i.n..

Digestion of cytokines by papain

Recombinant cytokines (4000 ng/ml rmIL-5, rmIL-13 or rmIL-2) in PBS were incubated with and without 1.2 mg/ml papain or heat-inactivated papain at 37°C for 30 min. The reaction was stopped by addition of a cocktail of proteinase inhibitors (cOmplete; Roche). The levels of cytokines in the solution were determined by ELISA, as described above.

Cytokine ELISA

Cytokine concentrations in culture supernatants and BALF were measured using mouse IL-4, IL-10 or TNF, and human/mouse TGF-β1 BD OptEIA ELISA sets (BD Biosciences), and mouse IL-2, IL-5, IL-6 and IL-13 ELISA Ready-Set-Go (eBioscience) kits, respectively.

Immunohistochemistry

For detection of IL-33-producing cells, paraffin sections were autoclaved in 0.01 M citric buffer (pH 6.0) at 121°C for 20 min, and then incubated with 5 μg/ml goat anti-mouse IL-33 polyclonal Ab (AF3626; R&D Systems Inc., Minneapolis, MN). After washing, the sections were incubated with Alexa 594-conjugated donkey anti-goat IgG Ab (Invitrogen) as the secondary Ab. The sections were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and then scanned with a confocal microscope (FV-1000; Olympus Corporation, Tokyo, Japan).

For detection of Treg cells and MCs, lungs of Foxp3-EGFP mice were harvested after papain inhalation and fixed with 4% paraformaldehyde in PBS for 1 h and then frozen in OCT compound (Sakura Finetek Japan Co, Tokyo, Japan). Frozen sections (5 μm thickness) were prepared using a cryostat (Leica, CM3050 S) and dried on treated slide glasses (Matsunami Glass, Osaka, Japan). The sections were permeabilized with 0.05% saponin and 0.3% BSA in PBS for 20 min at room temperature. After FcR blocking (anti-mouse CD16/CD32 mAb: 2.4G2) and treatment with 3% BSA in PBS, the sections were stained with Avidin-Texas Red (Invitrogen, A820) for detection of MCs (Kakurai et al., 2006) and anti-GFP Alexa 488 (Invitrogen, A21311) for detection of Treg cells with Hoechst 33258 (Invitrogen, H3569). For detection of IL-2 in MCs, frozen sections were prepared as described above and permeabilized, and FcR-blocked sections were stained with Avidin-Texas Red (Invitrogen, A820) and anti-mouse IL-2 Ab (Santa Cruz, sc-7896). Anti-rabbit IgG Alexa 488 (Invitrogen, A11008) was used as a 2^{nd} Ab with Hoechst 33258. After mounting with aqueous

mounting medium (Thermo, TA-030-FM), histological analyses were performed with a confocal laser microscope (LSM710; Carl Zeiss Microscopy).

T cell profile in BALF and LNs

BALF and thoracic LNs were collected at 24 h after the last inhalation of papain, heat-inactivated papain, IL-33 or saline. BALF cells were incubated with anti-mouse CD16/CD32 mAb (2.4G2: BD Pharmingen) in FACS buffer (HANKS Buffer containing 2% FCS) for 15 min on ice and then incubated simultaneously with APC-conjugated anti-mouse CD4 mAb (GK1.5: eBioscience) and PE anti-mouse CD25 mAb (PC61: eBioscience) for 40 min on ice. Thoracic LN cells were cultured with 1 μg/ml anti-mouse CD3 mAb (145-2C11; eBioscience) overnight and then stimulated with 0.1 μg/ml PMA (SIGMA) plus 1 μg/ml Ionomycin (SIGMA) in the presence of 1 μM monensin (SIGMA) for 6 h. The cells were then harvested and incubated with anti-mouse CD16/CD32 mAb (2.4G2: BD Pharmingen) in FACS buffer for 15 min on ice, followed by incubation with

APC-conjugated anti-mouse CD4 mAb for 40 min on ice. BAL cells and thoracic LN cells were fixed and then stained, respectively, with FITC-conjugated anti-mouse Foxp3 mAb (FJK-16s: eBioscience) and FITC-conjugated anti-mouse Foxp3 mAb plus PE anti-mouse IL-4 mAb (11B11: eBioscience) using an Anti-Mouse/Rat Foxp3 Staining Set (eBioscience). In the experiments using Foxp3-EGFP mice, BAL cells were incubated with PE-conjugated anti-mouse CD45, APC/Cy7-conjugated anti-mouse CD3 mAb, PE/Cy7-conjugated anti-mouse CD4 mAb and APC-conjugated anti-mouse CD25 mAb for 40 min on ice after FcR blocking. Intracellular molecular expressions of the transcription factor, Helios, and of cytokines in $CD4^+$ T cells or $CD4^+CD25^+$ T cells, and expression of Foxp3-EGFP in $CD45^+$ $CD3^+$ $CD4^+$ $CD25^+$ cells were analyzed on a FACSCalibur (Becton Dickinson), FACS Canto II (BD Bioscience) or MACSQuant (Miltenyi Biotech).

Detection of lung MCs by flow cytometry

Lungs were collected from mice 24 h after the challenge with papain. Single cell suspensions of the lungs were prepared as described elsewhere (Suto et al., 2006). Whole lung cells were stimulated with 0.1 μg/ml PMA plus 1 μg/ml Ionomycin in the presence of 1 μM monensin for 3 h, and then incubated with anti-mouse CD16/CD32 mAb for 15 min on ice and then incubated simultaneously with FITC-conjugated anti-mouse CD45 mAb, APC/Cy7-conjugated anti-mouse c-Kit mAb, and APC-conjugated anti-mouse FcεR1α mAb for 40 min on ice. After fixation and permeabilization, the cells were incubated with PE-anti-mouse IL-2 mAb for 30 min at 4 °C. The proportion of IL-2^+ cells in $CD45^+$ c-Kit^+ FcεR1α^+ MCs was analyzed on a FACS Canto II (BD Bioscience).

T cell preparation

Spleens and LNs (inguinal, brachial, axillary and submandibular) were collected and pooled for each animal group. For purification of naïve T cells,

$CD4^+CD25^-CD62L^+$ T cells (>97%) were isolated by CD62L-positive selection from $CD4^+CD25^-$ T cells using anti-CD62L beads (Miltenyi Biotec). For whole $CD4^+$ T cell purification, pooled spleen and LN cells were incubated with biotinylated anti-mouse CD8 (53-6.7), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (N418), CD49b (DX5), Ter119 (Ter119), γδTCR (GL3), FcεRIα (MAR-1) and c-kit/CD117 (2B8) for 20 min at 4°C. All antibodies were purchased from eBioscience. The cells were then washed and incubated with streptavidin particles plus DM (BD Bioscience) for 20 min at 4°C, the cell suspension was placed on an iMag magnetic field (BD Bioscience) and the negative fraction was collected (>95% $CD4^+$ T cells). $CD4^+$ $CD25^+$ Treg cells were approx. 5-10% of the purified whole $CD4^+$ T cells. For purification of Treg cells, whole $CD4^+$ T cells isolated as above were stained with APC anti-mouse CD4 and PE anti-mouse CD25 mAbs. Then $CD4^+$ $CD25^+$ T cells were sorted by FACS Aria II (BD Bioscience) (>95% $CD4^+$ $CD25^+$ T cells).

BMCMC culture and engraftment

BMCMCs were obtained by culturing mouse femoral bone marrow cells in the presence of 10 ng/ml rmIL-3 (PeproTech) for 6-8 weeks, at which time FACS analysis showed the cells to be >98% c-Kit^{hi}, FcεRIα^{hi}. After removal of IL-3 by washing, naïve BMCMCs (1 x 10^6 cells/well in a 24-well flat-bottom plate) were cultured with various concentrations of rhIL-33 for 24 h, and the culture supernatants were collected for measurement of cytokine concentrations. BMCMCs (1.0 x 10^7 cells) in 200 μl of saline were injected intravenously to 4 week-old *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Eight weeks after injection, the mice were used in experiments.

Detection, isolation and transfer of mouse NH cells

BALF was collected at 24 h after the last inhalation of papain, heat-inactivated papain or saline. BALF cells were incubated with anti-mouse CD16/CD32 mAb (2.4G2: BD Pharmingen) in FACS buffer (HANKS Buffer containing 2% FCS) for

15 min on ice and then incubated simultaneously with FITC-conjugated anti-mouse IL-1RL1/ST2 mAb (DJ8; MD Biosciences), PE conjugated lineage markers (anti-mouse CD3 mAb (145-2C11: eBioscience), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (1D3; BD Bioscience), CD49b (DX5), B220 (RA3-6B2), FcεRIα (MAR-1), Gr1 (RB6-8C5), NK1.1 (PK136; BD Bioscience) and γδTCR (GL3)), PE/Cy7-conjugated anti-mouse CD127 mAb (A7R34; BioLegend), APC-conjugated anti-mouse Sca-1 mAb (E13-161.7; BioLegend) and APC/Cy7-conjugated anti-mouse CD25 mAb for 40 min on ice. In the experiments using IL-13-dsTomato mice, BALF cells were incubated with FITC-conjugated lineage markers as above, APC/Cy7-conjugated anti-mouse Sca1 mAb and PE/Cy7-conjugated anti-mouse c-Kit mAb for 40 min on ice after FcR blocking. After addition of an equal volume of 2 μg/ml 7-aminoactinomycin D (7-AAD), IL-1RL1^{+} CD25^{+} NH cells in 7-AAD^{-} Lin^{-} Sca1^{+} CD127^{+} cells or IL-13-dsTomato^{+} NH cells in 7-AAD^{-} Lin^{-} Sca1^{+} c-Kit^{+} cells were analyzed on a FACS Canto II (BD Bioscience). IL-10R1 was stained with biotin-conjugated

anti-mouse IL-10R1 mAb (1B1.3a, BioLegend) and APC/Cy7-conjugated streptavidin, and its expression on Lin^- $CD90.1^+$ $Sca\text{-}1^-$ $IL\text{-}1RL1^+$ $CD25^+$ NH cells was determined by flow cytometry.

NH cells were isolated as below; In brief, mesenteries, from which intestinal and mesenteric LNs were separated, were minced and digested in DMEM containing 20 μg/ml Liberase DH (Roche) and 4% BSA. Lin- $c\text{-}Kit^+$ $Sca\text{-}1^+$ NH cells were enriched by AutoMACS (Miltenyi Biotec GmbH) and then sorted by a FACS Aria (BD Bioscience).

Mouse mast cell–T cell co-culture

T cells (1×10^6 cells/well (including approx. 5-10% $CD4^+$ $CD25^+$ Treg cells) in a 24-well plate) purified as described above were co-cultured for 3 days with naïve BMCMCs (5×10^5 cells/well) or BMCMCs sensitized with 1 μg/ml anti-DNP IgE (SPE-7: SIGMA) overnight (5×10^5 cells/well) in the presence and absence of 100 ng/ml cytokine (rmIL-1β, rmIL-13 or rhIL-33, obtained from PeproTech, and

rmIL-25, rmIL-33 or rmTSLP, obtained from R&D Systems), 1 μg/ml LPS (from *Salmonella enterica* serotype typhimurium: SIGMA), 1 μg/ml anti-DNP IgE (SPE-7), or 100 ng/ml DNP-HSA (SIGMA) with and without 40 μg/ml anti-mouse CD80 (16-10A1: BioLegend), CD86 (GL-1: BioLegend), PD-L1 (MIH5: eBioscience), OX40L (RM134L: eBioscience), ICOSL (HK5.3: eBioscience), ICAM-1 (YN1/1.7.4: BioLegend), FasL (MFL4: BioLegend) or 4-1BBL (TKS-1: BioLegend) mAbs, respectively. As controls, the same amount of hamster IgG, rat IgG2a or rat IgG2b (eBioscience) was added to the culture. To separate MCs and T cells, we used a Cell Culture Insert (0.4 μm pore size: BD Falcon) with $CD4^+$ T cells placed in the lower wells and BMCMCs placed in the upper wells in the presence and absence of rmIL-33 for 3 days. For detection of Th2 and Th17 cells, the cells were additionally stimulated with 0.1 μg/ml PMA (SIGMA) plus 1 μg/ml Ionomycin (SIGMA) in the presence of 1 μM monensin (SIGMA) for 6 h. The cells were harvested, subjected to FcR blocking using anti-mouse CD16/CD32 mAb and then incubated for 40 min on ice with APC anti-CD4, PE

anti-mouse CD25 (PC61: eBioscience) and/or PE-Cy5 anti-c-kit (2B8: eBioscience) mAbs in FACS buffer for detection of $Foxp3^+$ cells, with APC-Cy5 anti-CD4, APC anti-CD25 and PE-Cy5 anti-c-kit mAbs in FACS buffer for detection of $Foxp3^+$ $Helios^+$ cells, and with APC anti-CD4 and PE-Cy5 anti-c-kit mAbs in FACS buffer for detection of Th2 and Th17 cells. The cells were then fixed and stained with FITC-conjugated anti-mouse Foxp3 mAb or PE-conjugated anti-mouse/human Helios mAb (22F6, BioLegend) using an Anti-Mouse/Rat Foxp3 Staining Set (eBioscience), or with Alexa Fluor 488 anti-mouse IL-17 (eBio17B7, eBioscience), PE anti-mouse IL-4 (11B11, BD Bioscience) and PE anti-mouse IL-13 (eBio13A, eBioscience) mAbs using Perm/Wash Buffer (BD Biosciences). Foxp3 and Helios expression in c-kit-negative $CD4^+CD25^+$ T cells, and IL-4, IL-13 and IL-17 expression in c-kit-negative $CD4^+$ T cells, were analyzed on a FACSCalibur (Becton Dickinson), FACS Canto II (Becton Dickinson) or MACSQuant (Miltenyi Biotech).

Adoptive transfer of Treg cells

$CD4^+CD25^+$ Treg cells were isolated from spleen cells or co-cultures of mast cells and T cells in the presence of IL-33 as described above by using a $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec). The isolated $CD4^+CD25^+$ Treg cells (1×10^7 cells) were injected intravenously into mice. On the day after transfer, the mice were intranasally administered papain or IL-33, as described above.

Human mast cell–T cell co-culture

Peripheral blood-derived cultured mast cells were obtained as described previously (Saito et al., 2006). $CD4^+$ T cells were enriched from peripheral blood mononuclear cells of healthy donors using a human $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. Human cells were collected with the approval of the Ethical Review Board of the National Research Institute for Child Health & Development in Japan. The $CD4^+$ T cells (2×10^6

cells; containing approx. 1-2% of $CD4^+$ $CD25^+$ Treg cells) were co-cultured with and without mast cells (5 x 10^5 cells) in the presence and absence of 100 ng/ml rhIL-33 for 4 days. The co-cultures of T cells and mast cells used cells obtained from the same donor. Then the cells were harvested and incubated with APC anti-human CD4 (RPA-T4: BD Pharmingen) and PE anti-human CD25 (M-A251: BD Pharmingen) mAbs for 40 min on ice after FcR blocking (Miltenyi Biotech). The cells were then fixed and incubated with FITC anti-human Foxp3 mAb (PCH10: eBioscience) or isotype-matched control rat IgG2a (BD Pharmingen), and Foxp3 expression in $CD4^+CD25^+$ T cells was analyzed by FACSCalibur, as described above.

Human NH cell-Treg cell co-culture

Human peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation. Lin- IL-$7R^+$ $CRTH2^+$ $CD161^+$ cells were enriched by AutoMACS (Miltenyi Biotec GmbH) and then sorted with a FACS Aria (BD Bioscience). For

flow cytometric sorting, cells were stained with the following antibodies: FITC-conjugated lineage markers (anti-human CD1a (HI149), CD3 (OKT3), CD4 (RPA-T4), TCR$\alpha\beta$ (IP26), TCR$\gamma\delta$ (B1), CD11c (3.9), CD14 (HCD14), CD19 (HIB19), CD34 (581), CD94 (DX22), CD56 (HCD56), CD123 (6H6), FcεR1α (AER-37), CD303 (201A), Alexa Flour 488-conjugated anti-human CD16 (3G8), PE-Cy7-conjugated anti-human CD127 (A019D5), PerCP-Cy5.5-conjugated anti-human CD161 (HP-3G10), Brilliant Violet 421-conjugated anti-human CD117 (104D2), Brilliant Violet 510-conjugated anti-human CD45 (HI30) (all from Biolegend), and/or Alexa Flour 647-conjugated anti-human CD294 (BM16; BD Bioscience). Freshly isolated Lin^- IL-$7R^+$ $CRTH2^+$ $CD161^+$ cells were cultured in Yssel's Medium supplemented with 1% Human AB serum, together with irradiated allogeneic PBMCs, irradiated JY EBV-transformed B cells, 1 μg/ml PHA and 100 U/ml rhIL-2. Cells were sorted again with a FACS Aria before use.

$CD4^+$ $CD25^{high}$ $CD127^{dim/-}$ Treg cells were enriched from PBMCs of healthy donors using a human $CD4^+$ $CD25^+$ $CD127^{dim/-}$ regulatory T cell Isolation Kit II

(Miltenyi Biotec) according to the manufacturer's instructions and then sorted on a FACS Aria. For flow cytometric sorting, cells were stained with the following antibodies (all from BioLegend): FITC-conjugated anti-human CD3 (OKT3), PE-Cy7-conjugated anti-human CD127 (A019D5), APC-conjugated anti-human CD25 (BC96) and Brilliant Violet 510-conjugated anti-human CD4 (OKT4).

NH cells (1 x 10^4 cells/well) in 96-well round-bottom plates were co-cultured with different numbers of autologous Treg cells in the presence of 10 U/ml rhIL-2 and 50 ng/ml rhIL-33 at 37°C for 3 days. After 3 days, the cells were harvested and sorted again with a FACS Aria as described above. cDNAs were amplified directly from cells using a Whole Transcriptome Amplification Kit (Takara). The expression levels of IL-5 and IL-13 were determined by quantitative PCR.

Mouse mast cell-lung epithelial cell co-culture

For lung epithelial cells (ECs), mouse lungs were digested, and single cell suspensions of the lungs were prepared as described above. The lung cells (5

$\times 10^6$ cells/well) were plated into a 24-well plate at 37°C for 3 hours. Floating cells in the culture then were removed, and the wells were gently washed with warm PBS. The lung cells were co-cultured with BMCMCs (2×10^6 cells/well) in the presence of 5 μg/ml papain or heat-inactivated papain (data not shown) with 1 μM monensin at 37°C for 6 hours. Then, cells were collected and put onto a slide glass by a cytocentrifuge (Cytospin). After fixation with PFA, IL-2 was detected by IHC as described above.

Quantitative PCR

Lungs were collected from mice 24 hours after the last inhalation of papain or heat-inactivated papain. The total RNA in the lungs was purified, and expression of IL-33 mRNA in the lungs was determined by quantitative PCR, as described previously (Oboki et al., 2010).

Supplemental References

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